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Development of an in vitro-based potency assay for anthrax vaccine☆

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Abstract

The potency assay currently used to evaluate consistency of manufacture for the anthrax vaccine is contingent upon meeting specified parameters after statistical analysis of the percent survival and time to death of vaccinated guinea pigs after challenge with spores of a virulent strain of *Bacillus anthracis*. During the development of a new anthrax vaccine based upon recombinant protective antigen (rPA) adsorbed to aluminum hydroxide gel (Alhydrogel), we found that the serological response of female A/J mice, as measured by a quantitative anti-rPA IgG ELISA, may be an effective method to monitor a manufacturer's consistency for rPA-based vaccines. An advantage of the proposed in vitro-based potency assay is that it will not need stringent biosafety containment measures as required by the current guinea pig potency assay. Published by Elsevier Ltd.

Keywords: *Bacillus anthracis*; Vaccine; Potency assay

1. Introduction

Various potency tests have been developed to measure consistency in the quality of bacterial vaccines for human use during the manufacturing process. Potency tests include (i) measuring the biological activity of the immunogen in a living system (bioassay), as in determining protection against challenge, sero-conversion after vaccination, toxin neutralizing activity of antibodies, and passive protection; or (ii) biochemical analysis of the immunogen, such as measuring molecular size, purity, quantity, and preservation of specific epitopes [1,2]. The current vaccine licensed for use in the United States to protect humans against anthrax, anthrax vaccine adsorbed Biothrax (AVA Biothrax, also known as AVA or MDPH-PA) is prepared by adsorbing filtered culture supernatant fluids of the V770-NP1-R strain of *Bacillus anthracis* to aluminum hydroxide gel (Alhydrogel). In the United Kingdom, the human-use anthrax vaccine, alum-precipitated antigen (APA), is prepared by adsorbing filtered culture supernatant fluids of the $34F_2$ Sterne strain of *B. anthracis* to potassium aluminum sulfate (alum) [3]. The current potency assays for both AVA Biothrax and APA are based upon protection of guinea pigs vaccinated against a parenteral *B. anthracis* spore challenge. The Food and Drug Administration recently approved the relative potency assay for AVA Biothrax. In this test, guinea pigs are inoculated subcutaneously with one of four dilutions of either a test vaccine or reference vaccine and subsequently challenged intradermally 14 days later with spores of a virulent strain of *B. anthracis*. Dilutions of the challenge are also tested in an LD_{50} assay as an internal control for the challenge. Vaccine lots must pass the acceptance criteria of the relative potency model determined by statistical analysis of the percent survival and time to death data between the test lot and the reference lot for lot release. In addition, an acceptable challenge dose, as determined by the LD_{50}, must be measured. Biochemical analysis of protective antigen (PA) present in AVA Biothrax or APA after it is adsorbed to the adjuvant requires desorption from the adjuvant. Desorbing material on Alhydrogel can be accomplished by suspending AVA Biothrax in carbonate buffer pH 9 [4], whereas the material associated with alum can be released by dissolving the adjuvant in citric acid [5]. The presence of undefined bacterial and medium proteins, which are present in the

☆ Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the US Army.

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14. ABSTRACT

The potency assay currently used to evaluate consistency of manufacture for the anthrax vaccine is contingent upon meeting specified parameters after statistical analysis of the percent survival and time to death of vaccinated guinea pigs after challenge with spores of a virulent strain of Bacillus anthracis. During the development of a new anthrax vaccine based upon recombinant protective antigen (rPA) adsorbed to aluminum hydroxide gel (Alhydrogel), we found that the serological response of female A/J mice, as measured by a quantitative anti-rPA IgG ELISA, may be an effective method to monitor a manufacturer's consistency for rPA-based vaccines. An advantage of the proposed in vitro-based potency assay is that it will not need stringent biosafety containment measures as required by the current guinea pig potency assay.

15. SUBJECT TERMS

Bacillus anthracis, anthrax, vaccine, potency, in vitro

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culture supernatants, are also released from the adjuvant. Thus, to further analyze PA, it should be affinity purified from the desorbed protein mixture.

Vaccine preparations that contain PA, a component of the exotoxins from *B. anthracis*, can protect laboratory animals (mice, guinea pigs, rabbits, non-human primates) against intoxication or lethal anthrax infection [6–8]. PA is the component of the anthrax exotoxins that binds to cellular receptors [9] and undergoes proteolysis by the cell surface protease furin [10]. Alternatively, PA is cleaved by a serum protease(s) [11] before binding to the cell receptor. The resulting cell-bound PA63 heptamers [12] competitively bind lethal factor (LF) or edema factor (EF), forming lethal toxin (LeTx) and edema toxin [13], respectively, also promote internalization of LF and EF into the cytosol [14]. Previous studies demonstrated that a quantitative anti-PA IgG ELISA and toxin neutralizing antibody (TNA) assay served as immunological correlates to immunity in New Zealand white rabbits previously inoculated with AVA [15]. Passive protection studies with anti-PA antibodies demonstrated the significant role that antibodies play in protecting laboratory animals against infection [16–19]. Current studies have focused on development of the next-generation anthrax vaccine based upon recombinant PA (rPA) administered with Alhydrogel or alum. In this report, we present data supporting a new potency assay based upon the serological response of female A/J mice, as measured by a quantitative anti-rPA IgG ELISA, to evaluate a candidate next-generation anthrax vaccine based upon rPA adsorbed to Alhydrogel.

2. Materials and methods

2.1. Animals

For the potency assay, we used female A/J and CBA/J mice (Charles River Laboratories) 6–8 weeks old at the start of each experiment. To produce ascites fluids, we ordered female Balb/c mice (Charles River Laboratories) at 6–8 weeks old. The animals received food and water ad libitum. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2. Inoculation of mice

Before vaccine formulation, rPA was dialyzed against Dulbecco's phosphate buffered saline (DPBS) without calcium or magnesium. Purified rPA was manufactured as a GMP lot by the Biopharmaceutical Production Facility at NCI-FCRC (Frederick, Md.). It was expressed in a *B. anthracis* background [20,21] and purified by a modification of a procedure reported by Farchaus et al. [22]. Adsorption of rPA to Alhydrogel was allowed to occur at 4 °C for >2 h before inoculation of animals. Vaccine preparations were tested by two methods. First, different amounts of rPA protein were adsorbed to a fixed concentration of aluminum hydroxide adjuvant (Alhydrogel; 2% Al_2O_3; HCL Biosector (formerly Superfos Biosector) Frederikssund, Denmark) at 1.0 mg aluminum per ml final concentration. Mice were injected with 0.1 ml intramuscularly (i.m.). Four experiments were conducted with this formulation in A/J mice. Two experiments consisted of mice inoculated with 100, 31.6, 10, 3.2, or 1 μg of rPA protein and two experiments consisted of mice inoculated with 3.2, 1.0, 0.32, 0.1, or 0.032 μg of rPA protein. Only one experiment was conducted using CBA/J mice with 100, 31.6, 10, 3.2, or 1 μg of rPA adsorbed to Alhydrogel. Second, A/J mice were injected i.m. with 0.2 ml volumes of half-log serial dilutions of a vaccine preparation from a fixed starting concentration of rPA protein, either 31.6 μg of rPA (two experiments) or 10 μg of rPA (one experiment), and 0.2 mg of Alhydrogel. In addition, A/J mice were injected i.m. with 0.2 ml of AVA Biothrax lot FAV063 (BioPort Corporation, Lansing, MI). Control groups were injected with DPBS containing Alhydrogel at 1.0 mg of aluminum per ml final concentration. Twenty mice per test group were injected and sera obtained weekly on alternate groups of 10 mice via the retro-orbital sinus. Final bleeds were performed on all 20 mice in each group.

The serological responses of A/J mice to a booster inoculation were also examined. Mice were inoculated i.m. on weeks 0 and 4 with 0.1 ml of half-log concentrations of rPA (1, 0.32, 0.10, 0.032, and 0.01 μg) adsorbed to Alhydrogel at 1.0 mg of aluminum per ml final concentration. Sera were collected every week from alternate groups of 10 mice and tested in the quantitative anti-rPA ELISA and TNA assay.

2.3. Preparation of standards for ELISA and TNA assay

Standards for the quantitative anti-PA IgG ELISA and TNA assay were obtained from ascitic fluids prepared in Balb/c mice based upon the protocol published by Lacy and Voss [23]. The procedure used called for scheduled injections of pristine-primed Balb/c mice with antigen either emulsified with Freund's complete adjuvant (for preparation of the ELISA standard) or adsorbed to Alhydrogel (for the TNA assay standard) followed by injection of Sp2/0-Ag14 myeloma cells. The use of Freund's complete adjuvant optimized the development of a high-titer response against PA, whereas Alhydrogel optimized the expression of antibodies that resulted in high TNA assay titers (personal observations) and more closely resembled vaccine formulation. Mice were injected intraperitoneally (i.p.) with 0.5 ml of 2,6,10,14-tetramethylpentadecane (pristane, Sigma, St. Louis, MO) on day 0. Ten, seventeen, and thirty-eight days later, mice were injected i.p. with 50 μg of rPA either mixed 1:1 with Freund's complete adjuvant (0.2 ml) or adsorbed to

Alhydrogel (0.5 mg aluminum per 0.5 ml). On day 42, mice were injected i.p. with 1×10^6 of Sp2/0-Ag14 myeloma cells. Ascitic fluid was collected from the mice as it was produced. The pooled ascitic fluids were centrifuged at $25{,}000 \times g$ and passed through 0.2 μm filters.

The ascitic fluids collected for preparation of the ELISA standard were diluted 1:1 with 10 mM sodium phosphate, 138 mM NaCl, 2.7 mM KCl, pH 7.4 (PBS) before passage over rPA bound to Affi-Gel 15 resin (BioRad Laboratories, Hercules, CA). Bound antibody was eluted with 50 mM glycine/HCl, 10% ethylene glycol, pH 2.5, neutralized by adding 1 M Tris–HCl, pH 9.0, and dialyzed against PBS. The antibody was then passed over HiTrap Protein G (Pharmacia-Biotech, Piscataway, NJ) and the bound antibody was eluted with 0.1 M glycine/HCl, pH 2.7 and neutralized by adding 1 M Tris–HCl, pH 9.0. The antibody was dialyzed against PBS, filtered through 0.22 μm filters, and aliquots were frozen (−70 °C). Protein concentration was determined using the BioRad microplate protein assay (BioRad Laboratories). The seven concentrations of the affinity-purified anti-rPA IgG that yielded a linear response that were selected for use as standards in the ELISA ranged from 4.35 to 34.8 ng IgG per ml, which corresponded to dilutions of 1:400,000 to 1:50,000. The protein content of the affinity-purified anti-rPA IgG was measured at 1.74 mg/ml and the quantitative anti-PA IgG ELISA measured the concentration at 1.81 mg of anti-PA IgG per ml. The endpoint anti-PA ELISA titer for this material was 3.6×10^5 at an absorbance value of 0.200 as calculated by linear regression analysis (StandardCurve!Plus; ChemSW, Fairfield, CA).

Seven dilutions of the pooled ascitic fluids selected for use as standards for the TNA assay (mouse polyclonal anti-rPA/Alhydrogel ascites), ranging from 1:1500 to 1:4300, gave a linear response in the TNA assay. When this ascites was tested in the quantitative anti-rPA IgG ELISA, it had a titer of 1.08 mg anti-PA IgG per ml and an anti-PA ELISA endpoint titer of 4.4×10^5 at an absorbance value of 0.200 as calculated by linear regression analysis (StandardCurve!Plus).

2.4. Quantitative anti-rPA IgG ELISA

Microtiter plates (Immulon IIB, Dynex Technologies, Chantilly, VA) were coated with rPA at 100 ng per well in 100 μl volumes. After incubating overnight at 4 °C, the plates were washed three times with PBS, 0.1% Tween 20 (PBST). Samples, diluted in PBST containing 5% non-fat dry milk (PBSTM), were added to the wells and the plates were incubated for 1 h at 37 °C. The plates were washed three times in PBST, HRP-conjugated goat anti-mouse IgG (Kirkegaard & Perry, Gaithersburg, MD) was added to the wells, and the plates were incubated for 1 h at 37 °C. The plates were washed three times with PBST, rotated 180° and washed again three times with PBST before adding substrate (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt;ABTS; Kirkegaard & Perry) to the wells and incubating the plates at 37 °C. Absorbance readings were obtained using a BioTek 312e microplate reader (BioTek, Winooski, VT) after 20–30 min incubation. Concentrations of the samples were calculated from the standard curve by using BioTek KC4 software. All samples were tested in triplicate. Acceptance criteria included (i) the r^2 for the standard curve (seven separate dilutions) had to be ≥0.9700; (ii) the coefficient of variation (%CV) for the triplicate absorbance readings of the standards and samples had to be ≤20%; (iii) no more than one outlier, as identified by the Dixon Gap Test [24], could be removed from the standard curve; and (iv) if the %CV of the first sample dilution that could be read directly from the standard curve was >20%, the next sample dilution that could be read directly from the standard curve was used. If the sample was negative (below the limit of quantitation), the acceptance criteria for the standard were disregarded and the concentration of the sample was extrapolated from the standard curve, if possible, or assigned a value of 0.1 μg/ml. This value was determined by multiplying the lowest standard concentration (4.35 ng/ml) by the highest dilution tested (1:50) and dividing by two.

2.5. TNA assay

Ninety-six well plates (Costar; Corning Incorporated, Corning, NY), were seeded with 6×10^4–7×10^4 J774A.1 cells per well in 200 μl volumes 18–22 h before testing. Cells were maintained in Dulbecco's Minimal Essential Medium (D-MEM) containing 7.5% heat-inactivated fetal bovine serum, 4 mM glutamine, and 100 units of Penicillin G and 100 μg of streptomycin per ml (D-MEM complete). Standards and serial two-fold dilutions of samples were preincubated with LeTx (100 ng of rPA per ml and 50 ng of LF per ml, final concentrations) in a humidified incubator set at 37 °C, 5% CO_2 for 1 h. Dilutions of sample and LeTx were prepared in D-MEM complete containing 25 mM Hepes. Medium was removed from wells containing the J774A.1 cells and replaced with 100 μl per well of the sample or standard mixed with LeTx. After incubating for 4 h at 37 °C, 5% CO_2, 25 μl of 3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT, Sigma) at 5 mg/ml of PBS was added to each well. After incubating at 37 °C, 5% CO_2 for 2 h, the cells were lysed, and the precipitate was dissolved by adding 100 μl per well of solubilization buffer which consisted of 10% SDS in 50% dimethylformamide, pH 4.8 [25]. Absorbance readings were obtained using a BioTek 312e microplate reader (BioTek) at a wavelength of 570 nm less the reference wavelength at 690 nm after an overnight incubation at 37 °C. Each dilution of sample and standard was tested in triplicate. Nine wells contained only medium and served as medium controls. Three LeTx wells contained only LeTx and served as blanks. Acceptance criteria included (i) the r^2 for the standard curve had to be ≥0.9700; (ii) the standard curve had to contain at least five contigu-

ous standards; and (iii) the coefficient of variation (%CV) for the triplicate absorbance readings for standards and samples had to be ≤25%. The acceptance criteria for the standard were ignored if the sample was negative. The percent neutralization (P) for each dilution of sample and standard was determined by calculating $P = (\text{sample mean} - \text{LeTx mean})/(\text{medium control mean} - \text{LeTx mean}) \times 100$. Data were graphed using the logit-log statistical model, plotting $\text{Ln}(P/100 - P)$ on the *y*-axis and the $\log_{10}$ reciprocal of the dilution on the *x*-axis. The midpoint for each curve was calculated by $\text{Ln}^{(\text{intercept/slope})}$. Titers were expressed as either the reciprocal of the dilution of sample that neutralized 50% of the LeTx cytotoxicity (ED_{50}) or as the ratio between the sample ED_{50} and standard ED_{50} (RED_{50}). If titers for negative samples could not be calculated from the curve (i.e. below limits of quantification (BLQ)), they were arbitrarily assigned ED_{50} values of 1 or RED_{50} values of 0.0001 for reporting purposes.

2.6. Statistical analysis

No outliers were identified in ELISA or TNA assay titer variables. After $\log_{10}$ transformations were applied, the dependent variables met assumptions of normality and homogeneity of variance. ELISA titers that were BLQ were set to 0.1 μg IgG per ml. TNA assay ED_{50} and RED_{50} values that were BLQ were set to 0.0001 and 1, respectively. Pearson correlation coefficients were calculated between ED_{50} and RED_{50} values. Mixed model analysis of variance (ANOVA) was used to compare titers between experiments over concentration and time. All analyses were conducted using SAS Version 8.2 (SAS Institute Inc., SAS OnlineDoc, Version 8, Cary, NC, 2000).

3. Results

3.1. Serological response of A/J mice inoculated with rPA at fixed amounts of Alhydrogel

A/J mice were injected i.m. with half-log concentrations of rPA (100–0.032 μg) formulated with a fixed concentration of Alhydrogel (100 μg of aluminum) in 0.1 ml volumes. The weekly quantitative anti-rPA IgG ELISA titers for A/J mice injected with 100 μg of rPA/Alhydrogel showed that the peak geometric mean anti-PA IgG ELISA titer of 2179 μg anti-rPA IgG per ml occurred at week 6 and leveled off at about 1400 μg anti-rPA IgG per ml (Fig. 1). The geometric mean quantitative anti-PA IgG ELISA titers and standard errors for all test groups for weeks 3–6 are shown in Fig. 2. Regression equations were evaluated for the ability to predict titer from dose at weeks 2–8. The best-fitting regression model was a quadratic model that predicted week 5 titer from dose, $F(2, 147) = 92.12$, $P < 0.0001$; (adjacent $r^2 = 0.5502$). Week 5 quantitative anti-PA IgG ELISA titer was linear between 2.5 $\log_{10}$ of rPA (100 μg to 0.32 μg of rPA). An upper plateau anti-PA ELISA titer response in A/J mice appeared to be near the highest rPA concentration tested (100 μg). Concentrations of 0.032 μg of rPA generated an ELISA titer of 77 μg of anti-rPA IgG per ml at week 5.

The weekly ED_{50} TNA assay titers for A/J mice injected with 100 μg of rPA/Alhydrogel showed that the peak ED_{50} TNA assay titer of 2370 occurred at week 7, 1 week after the peak ELISA titer, and leveled off at a ED_{50} TNA assay titer of about 2000 (Fig. 1). Unlike the ELISA antibody response, the TNA assay response did not tend toward a linear dose response except at the lowest doses, between 0.32 and 0.032 μg of rPA (Fig. 3). Thus, the dose of rPA was not

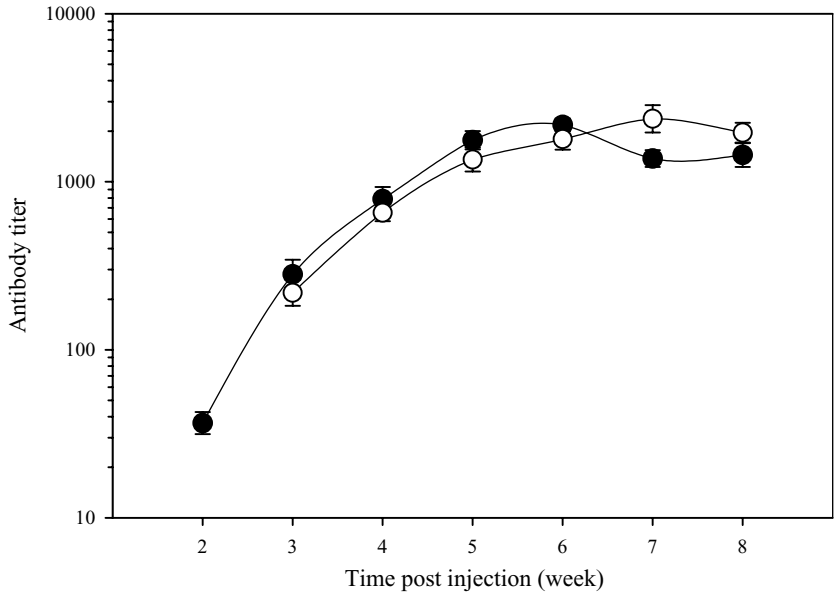

Fig. 1. Kinetics of the quantitative anti-rPA IgG ELISA and ED_{50} TNA assay titers in A/J mice inoculated with 100 μg of rPA formulated with Alhydrogel. Quantitative anti-rPA IgG ELISA titer ((●); μg anti-rPA IgG per ml) and ED_{50} TNA assay titer ((○); ED_{50}).

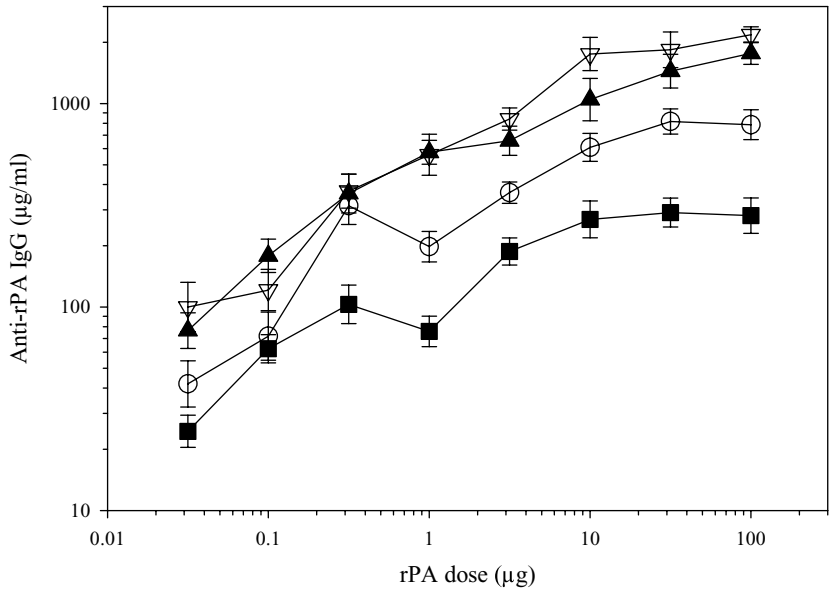

Fig. 2. Quantitative anti-rPA IgG ELISA titer of A/J mice inoculated with a single injection of various concentrations of rPA adsorbed to a fixed concentration of Alhydrogel. Sera were tested on week 3 (■), week 4 (○), week 5 (▲), and week 6 (▽).

a good predictor of ED_{50} titer at any week. Concentrations of 0.032 μg of rPA generated an ED_{50} TNA titer of 94 at week 5. The correlation between ED50 and RED50 values over weeks 2–8 was 0.9874 (Pearson r, $P < 0.0001$).

3.2. Serological response of CBA/J mice inoculated with rPA at fixed amounts of Alhydrogel

CBA/J mice that had been inoculated with various concentrations of rPA formulated to a fixed concentration of Alhydrogel (100 μg of aluminum) responded with significantly lower quantitative anti-rPA IgG ELISA and ED_{50} TNA assay titers than A/J mice that had been also inoculated with a similar vaccine preparation, $F(1, 946) = 316.31$, $P < 0.0001$. The kinetics of the quantitative anti-rPA IgG ELISA and ED_{50} TNA assay titers for CBA/J mice inoculated with 100 μg of rPA (Fig. 4) showed that the ELISA and ED_{50} TNA assay titers were significantly lower than those for A/J mice (ELISA: $F(1, 186) = 142.11$, $P < 0.0001$, TNA: $F(1, 155) = 198.63$, $P < 0.0001$). The peak geometric mean anti-PA IgG ELISA titer occurred at about week 5 (296.1 μg of anti-rPA IgG per ml). In addition, for doses

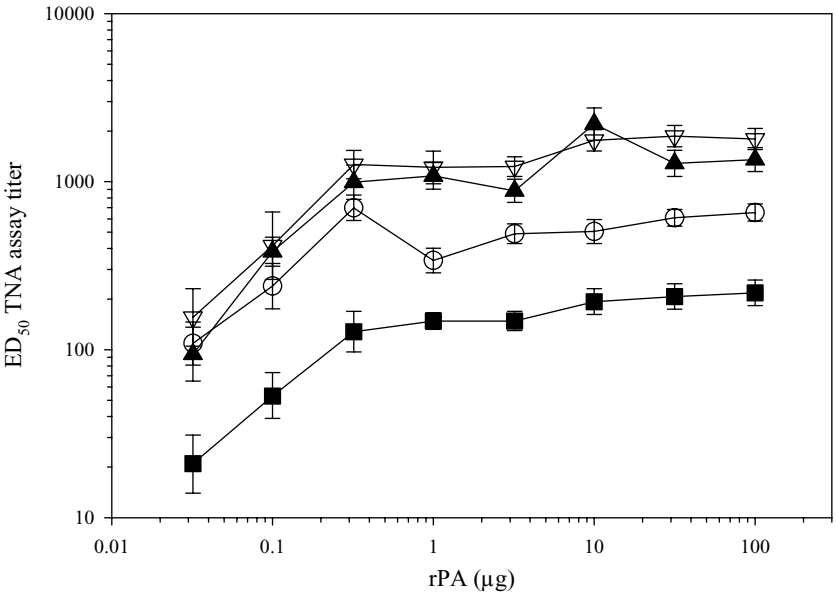

Fig. 3. ED_{50} TNA assay titers of A/J mice inoculated with a single injection of various concentrations of rPA adsorbed to a fixed concentration of Alhydrogel. Sera tested on week 3 (■), week 4 (○), week 5 (▲), and week 6 (▽).

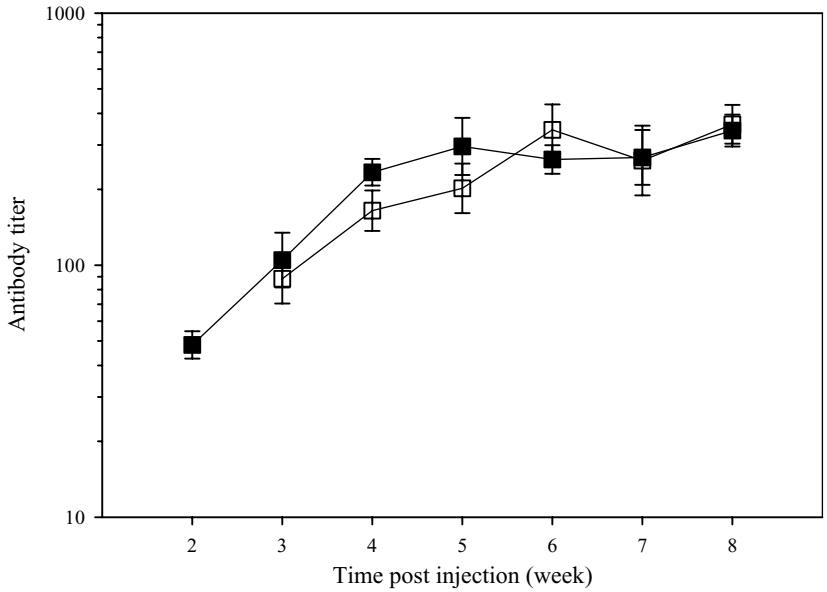

Fig. 4. Kinetics of the quantitative anti-rPA IgG ELISA and ED_{50} TNA assay titers in CBA/J mice inoculated with 100 μg of rPA formulated with Alhydrogel. Quantitative anti-rPA IgG ELISA titer (■; μg anti-rPA IgG per ml) and ED_{50} TNA assay titer ((□); ED_{50}).

between 10 and 100 μg of rPA, quantitative anti-PA IgG ELISA titers remained constant at about 300 μg of anti-rPA IgG per ml from week 5 until the last blood sample taken on week 8 (Table 1). Dose-dependent ELISA titers were only measured at concentrations of <10 μg of rPA beginning at week 5 (Table 1). The peak ED_{50} TNA assay titer occurred at week 6 (Fig. 4; ED_{50} of 362.1).

3.3. Serological response of A/J mice inoculated with diluted rPA vaccine or AVA

Two groups of A/J mice were inoculated i.m. with 0.2 ml of dilutions of either a rPA vaccine preparation at initial concentrations of 31.6 μg of rPA or 10 μg of rPA and containing 200 μg of aluminum per dose. A third group of A/J mice was inoculated with 0.2 ml of dilutions of AVA Biothrax, which contained 240 μg of aluminum per dose. The concentration of PA in AVA Biothrax was unknown. When change over concentration was compared between the 2 rPA groups at each week, results showed that there was no significant interaction between concentration and group. Therefore, the 2 rPA groups had parallel ELISA antibody responses, as is shown in Fig. 5. ELISA titers from mice inoculated with dilutions of a vaccine prepared from a starting concentration of rPA adsorbed to Alhydrogel declined much more rapidly over concentration than when a vaccine was prepared with a constant concentration of Alhydrogel ($F(5, 2214) = 73.86$, $P < 0.0001$). Similarly, ED_{50} TNA assay titers also dropped more rapidly over concentration when both rPA and Alhydrogel were diluted from a fixed starting concentration than when a constant concentration of Alhydrogel was used to prepare the vaccine at each protein concentration ($F(4, 1661) = 218.71$, $P < 0.0001$) (Table 2).

3.4. Serological response of A/J mice inoculated with two doses of rPA vaccine

Kinetics of the quantitative anti-rPA IgG ELISA and ED_{50} TNA assay titers of A/J mice inoculated i.m. with 0.1 ml of rPA vaccine at 1, 0.1, and 0.01 μg of rPA each containing

Table 1
Geometric mean anti-PA IgG ELISA titers and standard errors of CBA/J mice inoculated with various concentrations of rPA adsorbed to a fixed concentration of Alhydrogel

rPA (μg)	Weeks post-vaccination						
	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Geometric mean ELISA titer (μg IgG per ml) and standard error							
100	48.3 (1.13)	104.8 (1.28)	233.6 (1.13)	296.1 (1.30)	262.5 (1.14)	267.5 (1.28)	342.0 (1.16)
31.6	39.2 (1.21)	130.3 (1.10)	169.2 (1.27)	319.6 (1.22)	215.6 (1.32)	273.0 (1.22)	315.0 (1.16)
10	59.8 (1.22)	146.5 (1.10)	270.2 (1.32)	330.3 (1.17)	303.9 (1.34)	255.6 (1.17)	300.8 (1.16)
3.2	52.3 (1.25)	122.0 (1.13)	156.9 (1.31)	197.9 (1.16)	255.3 (1.38)	235.5 (1.20)	178.9 (1.18)
1	47.1 (1.17)	96.7 (1.13)	152.8 (1.17)	146.4 (1.21)	226.1 (1.24)	127.0 (1.26)	147.4 (1.17)

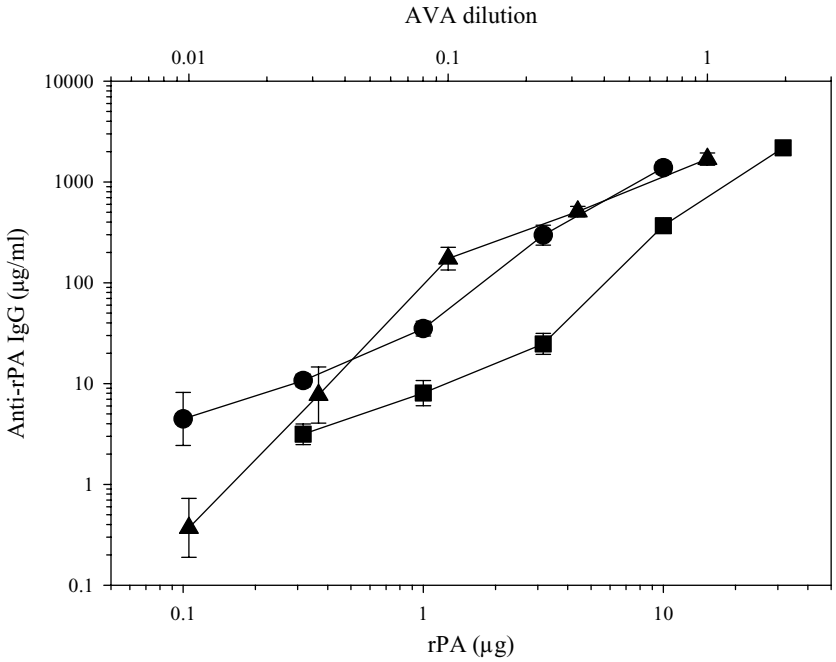

Fig. 5. Week 5 quantitative anti-rPA IgG ELISA titers of A/J mice inoculated with dilutions of rPA formulated with Alhydrogel or AVA. Starting concentration of rPA vaccine was at 31.6 μg (■) or 10 μg (●) and AVA was undiluted (▲).

Table 2
Geometric mean ED_{50} TNA assay titers and standard errors of sera from A/J mice inoculated i.m. with dilutions of a rPA vaccine formulated with Alhydrogel

rPA dose (μg)	Weeks post-vaccination					
	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8
Geometric mean TNA assay ED50 titers and standard errors						
31.6	431.6 (1.20)	1083.6 (1.22)	1548 (1.20)	1885.5 (1.19)	1499.2 (1.18)	2062.2 (1.11)
10	144.7 (1.25)	239.6 (1.29)	444.4 (1.23)	341.2 (1.24)	508.7 (1.20)	517.6 (1.19)
3.2	4.9 (1.46)	5.4 (1.48)	23.7 (1.61)	21.3 (1.39)	41.6 (1.54)	30.1 (1.33)
1	3.0 (1.34)	2.2 (1.32)	4.7 (1.52)	3.0 (1.33)	10.1 (1.58)	6.7 1.32)
0.32	1.6 (1.22)	1.7 (1.28)	1.3 (1.19)	1.9 (1.33)	1.9 (1.36)	2.6 (1.21)

100 μg of aluminum at 0 and 4 weeks are shown in Fig. 6a and b. For comparative purposes, the quantitative anti-rPA IgG ELISA and ED_{50} TNA assay titers for A/J mice that received a single dose of rPA vaccine at 1.0 μg of rPA are also plotted (Fig. 6a and b). When weekly titers were compared between the two rPA groups at each concentration, results showed that the two-dose rPA groups quantitative anti-rPA IgG ELISA titers were significantly higher than those of the single-dose rPA groups ($F(1, 848)$=134.69, $P < 0.0001$). Results also showed that the two-dose rPA groups ED_{50} TNA assay titers were significantly higher than those of the single-dose rPA groups ($F(1, 832) = 228.86$, $P < 0.0001$). For weeks 2–4, the two-dose 1 μg rPA group quantitative anti-rPA IgG ELISA titers were about 2.5-fold higher than those of the single-dose 1 μg rPA group, the titers of which were similar to the two-dose 0.1 μg rPA group (Fig. 6a). After the booster, the quantitative anti-rPA IgG ELISA titers for the two-dose 1 μg rPA and the two-dose 0.1 μg rPA groups were about eight-fold and two-fold higher, respectively, than those of the single-dose 1 μg rPA group (Fig. 6a). At weeks 3 and 4, the ED_{50} TNA assay titers for the two-dose 1 μg rPA group were about two-fold higher than those of the single-dose 1 μg rPA group, the titers of which were similar to the two-dose 0.1 μg rPA group. After the booster injection, the two-dose, 1 and 0.1 μg rPA groups were about 10-fold and 6-fold higher, respectively, than those of the single-dose 1 μg rPA group (Fig. 6b). Two inoculations with 0.01 μg of rPA resulted in weeks 6–8 an average ELISA titer of 177 μg of anti-rPA IgG per ml and ED_{50} titer of 728.

4. Discussion

A potency assay, as applied to immunobiologicals, refers to the test that is used to monitor the lot-to-lot consistency of the product being evaluated. Because knowledge of the

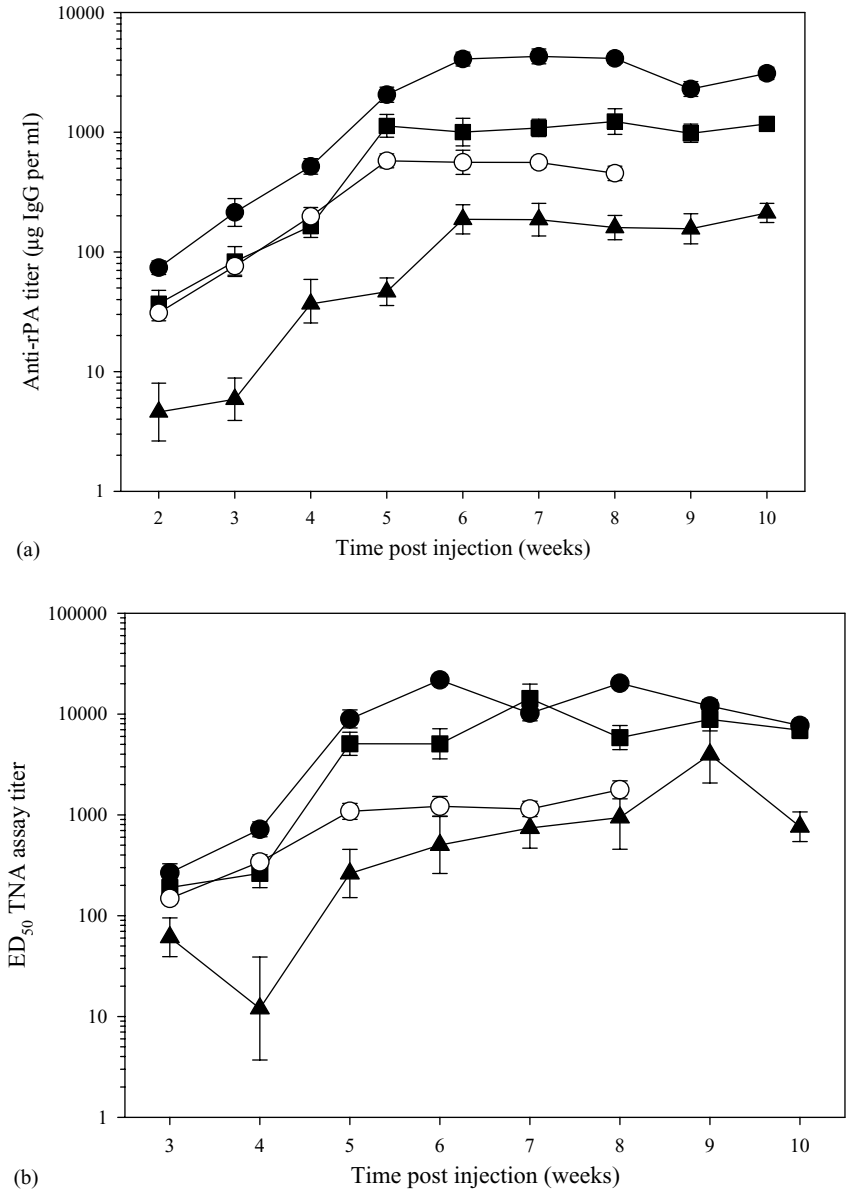

Fig. 6. (a) Kinetics of the quantitative anti-rPA IgG ELISA titer in A/J mice inoculated at 0 and 4 weeks with either 1 μg, 0.1 μg, or 0.01 μg of rPA formulated with Alhydrogel or a single dose at 1 μg of rPA. Two doses of rPA at 1 μg (●), 0.1 μg (■), or 0.01 μg (▲), and 1 dose of rPA at 1 μg (○). (b) Kinetics of the ED_{50} TNA assay titer in A/J mice inoculated at 0 and 4 weeks with either 1 μg, 0.1 μg, or 0.01 μg of rPA formulated with Alhydrogel or a single dose at 1 μg of rPA. Two doses of rPA at 1 μg (●), 0.1 μg (■), or 0.01 μg (▲), and 1 dose of rPA at 1 μg (○).

protection afforded by anthrax vaccines is limited [26] by reason of ethical constraints in evaluating efficacy in humans directly, surrogates are required as a means to predict human efficacy. Thus, the potency test selected should be such that it could function as a surrogate to predict the efficacy of the product in humans. Non-clinical potency assays established for bacterial vaccines are described under four federal regulatory documents and can be generally divided to include protection studies in animals, serological measurement of an immune response, characterization of the immunogenic epitope**s**, or biochemical assay of the antigen [1]. As part of pre-clinical experiments for a second-generation rPA vaccine, we evaluated the serological response of mice in a quantitative anti-PA IgG ELISA and TNA assay for use as an in vitro-based potency assay. The measurement of antibody levels in a bioassay may offer an alternative to the relative guinea pig potency assay that relies on meeting specified parameters after statistical analysis of percent survival and time to death of animals vaccinated with the test lot and reference lot of vaccine. The relative guinea pig potency assay also requires the use of a laboratory with biosafety containment levels that meet safety regulations to conduct research using virulent strains of *B. anthracis.*

Standards for the quantitative anti-PA IgG ELISA and TNA assay were prepared from ascitic fluids prepared in Balb/c mice based upon the protocol published by Lacy

and Voss [23]. Using this method, we were able to collect a large amount of ascites from fewer animals as compared to traditional methods of collecting serum. The procedure for producing ascitic fluids calls for scheduled injections of pristane-primed Balb/c mice with antigen emulsified 1:1 with Freund's complete adjuvant followed by Sp2/0-Ag14 myeloma cells. We prepared affinity-purified anti-PA IgG from ascitic fluids collected from Balb/c mice that were injected with 50 μg of rPA emulsified 1:1 with Freund's complete adjuvant. In addition, we also produced ascitic fluid in Balb/c mice by injecting 50 μg of rPA adsorbed to Alhydrogel. The ascitic fluid produced using rPA adsorbed to Alhydrogel was used as the standard for the TNA assay. Thus, we were able to develop high-titer ascites against rPA with either Freund's complete adjuvant or Alhydrogel using this procedure. When the ELISA and TNA assay were evaluated using sera from A/J mice, we found that the quantitative anti-PA IgG ELISA titer was a better predictor of concentration than the TNA assay titer. Week 5 ELISA titers between 0.32 and 100 μg of rPA increased with a corresponding increase in rPA dosage whereas the ED_{50} TNA assay titers did not show a corresponding increase with an increase in the concentration of rPA except between 0.032 and 0.32 μg of rPA. Also, the amount of rPA that was required in mice to attain maximal TNA assay titers (i.e. neutralizing antibody activity; 0.32 μg of rPA) was lower than what was needed to attain a maximal ELISA titer response (ca. 100 μg of rPA). We initially hypothesized that a potency assay like the TNA assay would provide a means of determining the functional antibody response to PA in neutralizing the cytotoxic effects of LeTx. Our findings in mice contrast with those of Reuveny et al. [19] who reported that the TNA assay may be useful as a surrogate marker for protection in guinea pigs. Alternatively, a competitive ELISA using monoclonal antibodies that define functional epitopes of PA may be informative [27].

Welkos and Friedlander [28], reporting on the susceptibility of inbred mouse strains against challenge with the Sterne strain of *B. anthracis*, classified A/J mice as susceptible ($LD_{50} = 2500$ spores) and CBA/J mice as resistant ($LD_{50} = 2 \times 10^7$ spores). We found that A/J and CBA/J mice differed in their quantitative anti-PA IgG ELISA titer and TNA assay titer responses after inoculation with rPA adsorbed to Alhydrogel. A/J mice had peak quantitative anti-PA IgG ELISA titers of about 2000 μg of IgG per ml and ED_{50} TNA assay titers of about 2000. The similarity between the maximum quantitative anti-rPA IgG titers and ED_{50} TNA assay titers may have been fortuitous with a single inoculation at that concentration of rPA and Alhydrogel. The close similarity between the two measured responses was not evident after a booster inoculation with rPA. Two inoculations with 1.0 μg of rPA at 0 and 4 weeks of A/J mice resulted in peak quantitative anti-PA IgG ELISA titers of about 4000 μg anti-PA IgG per ml and ED_{50} TNA assay titers of about 14,000. Booster injections of A/J mice of 1 μg of rPA resulted in higher titers than a single injection of 100 μg of rPA and resulted in about 10-fold higher ELISA and ED_{50} TNA assay titers compared to week 4 titers, a time when titers were still climbing and about 1–2 weeks before peak ELISA and ED_{50} TNA assay titers were measured in the single-dose experiments. On the other hand, CBA/J mice had peak quantitative anti-PA IgG ELISA titers of about 300 μg anti-PA IgG per ml and ED_{50} TNA assay titers of about 300. Welkos and Friedlander [29] have also reported differences in the anti-PA endpoint ELISA titer between A/J and CBA/J mice. Three doses of AVA at 2-week intervals resulted in anti-PA endpoint titers of 758,578 in A/J mice while CBA/J mice had titers of 100,000. It thus appears that CBA/J mice respond with titers lower than those from A/J mice, even after a booster inoculation.

In contrast to the quantitative anti-PA IgG ELISA titers that were measured from sera of A/J mice that had been inoculated with various concentrations of rPA adsorbed to a fixed concentration of Alhydrogel, we noted a more rapid drop in the quantitative anti-PA IgG ELISA titers from sera of A/J mice that had been inoculated with similar concentrations of PA but lower concentrations of Alhydrogel. The latter vaccine was prepared by diluting the vaccine from an initial, fixed concentration of rPA and Alhydrogel. This rapid decline in antibody titer may correspond with the dilution of rPA, but more likely with a decrease in the stimulation of the immune response in the animal due to the lower amount of adjuvant present in the inoculations. This is supported by experiments in rabbits that had significantly lower ELISA titers after being injected with soluble rPA compared with rabbits that had been injected with rPA adsorbed to Alhydrogel (personal observations). We observed a similar drop in ELISA titer that was measured with dilutions of the rPA vaccine in sera from mice that had been inoculated with dilutions of AVA Biothrax. The differences between the antibody responses of rPA and AVA Biothrax may be due to the presence of other bacterial and medium components, as well as formaldehyde, in AVA Biothrax.

As part of pre-clinical experiments for an rPA vaccine using the New Zealand white rabbit aerosol model, we found that a single dose of 100 μg of rPA protected 93% of rabbits against an aerosol challenge, and 50% survival, calculated by Probit analysis, was measured at 7.78 μg of rPA [30]. Our results suggest that a potency assay based upon the serological response of A/J mice, as measured at week 5 by a quantitative anti-rPA IgG ELISA between 100 and 0.32 μg of rPA, may be an effective method to monitor manufacturing consistency of a next-generation rPA-based vaccine.

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